

09/529172

PCT/IL 98/00487



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## REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

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International Application No. **PCT/IL 97 / 00328**

International Filing Date

**10 OCTOBER 1997**  
(10.10.97)
**ISRAEL PATENT OFFICE**  
**PCT International Application**

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference

(if desired) (12 characters maximum) **108236**

Box No. I - **TITLE OF INVENTION**  
**TRANSGENIC LEMNACEAE**

Box No. II **APPLICANT**

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)

**YEDA RESEARCH AND DEVELOPMENT CO. LTD.**  
 at the Weizmann Institute of Science  
 P.O.B. 95  
 Rehovot 76100  
 ISRAEL

☐ This person is also inventor.

Telephone No.

Facsimile No.

Teleprinter No.

State (i.e. country) of nationality:

**IL**

State (i.e. country) of residence:

**IL**

This person is applicant for the purposes of:

☐

all designated States

☒

all designated States except the United States of America

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the United States of America only

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the States indicated in the Supplemental Box

Box No. III **FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)**

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)

**EDELMAN, Meir**  
 20 Pinsker Street,  
 Rehovot 76308  
 ISRAEL

This person is:

☐

applicant only

☒

applicant and inventor

☐

inventor only (If this check-box is marked, do not fill in below.)

State (i.e. country) of nationality:

**IL**

State (i.e. country) of residence:

**IL**

This person is applicant for the purposes of:

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all designated States

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the United States of America only

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the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV **AGENT OR COMMON REPRESENTATIVE, OR ADDRESS FOR CORRESPONDENCE**

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:

☒

agent

☐

common representative

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)

**REINHOLD COHN AND PARTNERS**  
 P.O. Box 4060  
 Tel-Aviv 61040  
 ISRAEL

Telephone No.

**+972 3 5664455**

Facsimile No.

**+972 3 5606405**

Teleprinter No.

☐ Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

## Continuation of Box No. III FURTHER APPLICANTS AND/OR (FURTHER) INVENTORS

*If none of the following sub-boxes is used, this sheet is not to be included in the request.*

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)

PERL, Avihai  
2 Drubin Street,  
Rishon Le Zion-75231  
ISRAEL

This person is:

☐ applicant only

☒ applicant and inventor

☐ inventor only (If this check-box is marked, do not fill in below.)

State (i.e. country) of nationality:

IL

State (i.e. country) of residence:

IL

This person is applicant for the purposes of:

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Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)

FLAISHMAN, Moshe  
7 Phichman Street  
Ramat-Aviv, Tel-Aviv 69027  
ISRAEL

This person is:

☐ applicant only

☒ applicant and inventor

☐ inventor only (If this check-box is marked, do not fill in below.)

State (i.e. country) of nationality:

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BLUMENTHAL, Amnon  
21/3 Kavshei Ha'hermon Street  
Rehovot 76555  
ISRAEL

This person is:

☐ applicant only

☒ applicant and inventor

☐ inventor only (If this check-box is marked, do not fill in below.)

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Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)

This person is:

☐ applicant only

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## Box No.V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) (mark the applicable check-boxes; at least one must be marked):

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In addition to the designations made above, the applicant also makes under Rule 4.9(b) all designations which would be permitted under the PCT except the designation(s) of \_\_\_\_\_

The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. (Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)

Sheet No. ...4...

## Box No. VI PRIORITY CLAIM

Further priority claims are indicated in the Supplemental Box ☐

The priority of the following earlier application(s) is hereby claimed:

Country (in which, or for which, the application was filed)	Filing Date (day/month/year)	Application No.	Office of filing (only for regional or international application)
item (1)			
item (2)			
item (3)			

Mark the following check-box if the certified copy of the earlier application is to be issued by the Office which for the purposes of the present international application is the receiving Office (a fee may be required):

☐ The receiving Office is hereby requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) identified above as item(s):

## Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) (If two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used): ISA / EP

Earlier search Fill in where a search (international, international-type or other) by the International Searching Authority has already been carried out or requested and the Authority is now requested to base the international search, to the extent possible, on the results of that earlier search. Identify such search or request either by reference to the relevant application (or the translation thereof) or by reference to the search request:

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Date (day/month/year):

Number:

## Box No. VIII CHECK LIST

This international application contains the following number of sheets:

1. request : 4 sheets  
 2. description : 46 sheets  
 3. claims : 5 sheets  
 4. abstract : 1 sheets  
 5. drawings : 1 sheets

Total : 57 sheets

This international application is accompanied by the item(s) marked below:

1. ☐ separate signed power of attorney  
 2. ☐ copy of general power of attorney  
 3. ☐ statement explaining lack of signature  
 4. ☐ priority document(s) identified in Box No. VI as item(s)  
 5. ☒ fee calculation sheet  
 6. ☐ separate indications concerning deposited microorganisms  
 7. ☐ nucleotide and/or amino acid sequence listing (diskette)  
 8. ☐ other (specify):

Figure No. 1 of the drawings (if any) should accompany the abstract when it is published.

## Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

GALLILY, Tamar  
 Agent for REINHOLD COHN AND PARTNERS  
 P.O. Box 4060  
 Tel-Aviv 61040  
 ISRAEL

GALLILY, Tamar

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1. Date of actual receipt of the purported international application: 10 OCTOBER 1997 (10.10.97)	2. Drawings: <input checked="" type="checkbox"/> received: ALK <input type="checkbox"/> not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:	
4. Date of timely receipt of the required corrections under PCT Article 11(2):	
5. International Searching Authority specified by the applicant: ISA / EP	6. <input checked="" type="checkbox"/> Transmittal of search copy delayed until search fee is paid

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# PCT

## FEE CALCULATION SHEET Annex to the Request

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PCT/IL 97 / 00328

International application No.

10 OCTOBER 1997

Date stamp of the receiving Office

Applicant's or agent's  
file reference

108236

Applicant

YEDA RESEARCH AND DEVELOPMENT CO. LTD.

IL

US\$

### CALCULATION OF PRESCRIBED FEES

1. TRANSMITTAL FEE . . . . . IL 383 T

383

2. SEARCH FEE . . . . . US\$ 1,180 S

1,180

-International search to be carried out by EP  
(If two or more International Searching Authorities are competent in relation to the international application, indicate the name of the Authority which is chosen to carry out the international search.)

3. INTERNATIONAL FEE

#### Basic Fee

The international application contains 57 sheets.

first 30 sheets . . . . . US\$ 530 b<sub>1</sub>

530

27 x 10 = US\$ 270 b<sub>2</sub>  
remaining sheets additional amount

270

Add amounts entered at b<sub>1</sub> and b<sub>2</sub> and enter total at B . . . . . US\$ 800 B

800

#### Designation Fees

The international application contains all designations.

11 x 128 = US\$ 1,408 D  
number of designation fees amount of designation fee payable (maximum 11)

1,408

Add amounts entered at B and D and enter total at I . . . . . US\$ 2,208 I

2,208

(Applicants from certain States are entitled to a reduction of 75% of the international fee. Where the applicant is (or all applicants are) so entitled, the total to be entered at I is 25% of the sum of the amounts entered at B and D.)

4. FEE FOR PRIORITY DOCUMENT . . . . . P

5. TOTAL FEES PAYABLE

Add amounts entered at T, S, I and P, and enter total in the TOTAL box . . . . . IL 383/US\$ 3,388

383 3,388

TOTAL

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### MODE OF PAYMENT

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- 1 -

## TRANSGENIC LEMNACEAE

### FIELD OF THE INVENTION

The present invention relates to stably transformed plants, progeny thereof and products obtained from the cells or progeny. The invention further concerns methods for the genetic transformation of plants  
5 and more specifically to a method wherein *Agrobacterium* is used as the transforming vector.

### PRIOR ART

The prior art considered to be pertinent to the following  
10 disclosure is listed in the section entitled "*References*" before the claims.

### BACKGROUND OF THE INVENTION

Genetic transformation of plants is gradually beginning to play an important role in modern agriculture. Attempts are made to introduce  
15 heterologous DNA into plants in order to increase their resistance to viral infection, acquire or increase resistance to various herbicides, modulate ripening or decay times, increase the nutritional value of various plant products, bring them to produce pharmaceuticals, and produce various other chemical and biological molecules.

20 Commercial production of transgenic compounds in bacterial, yeast and mammalian cell systems is often beset by high capital investment costs in fermentation equipment and the necessity to eliminate prion or microplasmal components from the purified product. Recently, production of heterologous proteins and peptides (e.g.,  $\alpha$ -amylase, antibodies,  
25 enkephalins, human serum albumin) has been achieved in plants (Pen *et al.*,

1992, Miele, 1997). Potential advantages of transgenic plant systems are: lowered production costs of biomass and a reduction in the biohazard of contaminants in downstream processing of the products. Transgenic plants may thus be superior bioreactors for bulk enzymes in industry, purified  
30 products in medicine and orally active pharmaceuticals.

- 2 -

In order to transform plants to produce a desired product, the relevant gene, once identified and cloned, has to be introduced into the plant of interest so that the resulting plant is capable of passing the gene to its progeny. The methods of introduction proposed for this purpose include  
5 electroporation, microinjection, microprojectile bombardments, liposome fusion, *Agrobacterium* mediated transfer, and many others.

One of the most commonly used transforming vectors is *Agrobacterium*, which is a genus of plant pathogenic bacteria of the family *Rhizobiaceae*, which does not fix free nitrogen and usually produces gall and  
10 hairy roots in infected cells. Heterologous DNA is introduced into the *Agrobacterium* and through a process of transfection wherein genetic material from the *Agrobacterium* enters the plant's cell, genetic transformation of the plant takes place (Armitage *et al.*, 1992). *Agrobacterium* infects primarily dicotyledonous plants and infects monocotyledonous plants only  
15 at a very low yield (Armitage *et al.*, 1992).

One attempt to transform monocotyledonous plants was by a particle gun wherein the heterologous DNA is delivered by air or by helium into the plant or plant cell to be transformed. This technique has two main disadvantages: first, it is quite difficult to target the DNA particles to the  
20 meristematic zone wherein, for certain plants such as those of the family *Lemnaceae*, the transformation should take place in order to enable regeneration therefrom of a full transformed plant; second, even if the DNA particle enters the cell in the meristematic zone and reaches the nucleus thereof, the DNA does not usually integrate into the cell's chromosome and,  
25 thus after a few cell cycles the unintegrated heterologous DNA is lost, so that transformation by a particle gun is usually merely transient.

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It would have been highly desirable to provide a method for the genetic transformation of monocotyledonous plants which would result in stable transformation with a satisfactory yield.

30 One of the most commercially promising monocotyledons are the *Lemnaceae*, a widely distributed aquatic family of small (1-5 mm) plants.



The *Lemnaceae* excel in two characteristics potentially exploitable by the biotechnology industry: their extraordinary vegetative growth rates and a high tolerance for a spectrum of nutrients and toxic substances (Landolt and Kandeler, 1987). In the U.S.A., commercialization of *Lemnaceae* has centered around waste water management and animal feed (Culley *et al.*, 1981; Ngo, 1987). However, the use of mixed aquacultures and conventional technology has met with only moderate success. A different approach was taken in Israel, utilizing the *Lemna gibba* Hurfeish strain (Porath *et al.*, 1979). With its especially short root and high protein, carotenoid and iron content, this strain was cultivated under modern greenhouse conditions (4 tons harvested per acre per week; Tzora Biotechnology Inc., Kibbutz Tzora), and successfully marketed as a packaged vegetable product for the food industry. Notwithstanding the exceedingly high growth rates and the promising future of *Lemnaceae* as a potential food source, various attempts to genetically transform these plants, by a stable transformation method proved, to date, quite unsuccessful. The failure of transformation was due to the fact that *Lemnaceae* multiply vegetatively, daughter fronds arising from meristematic zones deep inside the mother frond. Thus, the meristem initial must be reached for stable transformation to take hold. Particle bombardment of *Lemnaceae*, the current state-of-the-art method used by several groups to obtain localized, transitory transformation events, was found by the inventors of the application ineffective in transformation of daughter fronds.

It would have been highly desirable to obtain *Lemnaceae* plants which are stably transformed with heterologous DNA of interest and to use such transformed plants for the production of chemical and biological products.

## GENERAL DESCRIPTION OF THE INVENTION

In the following description, the term "*transformation*" will be used to denote the introduction of a transforming DNA into plant cells or

- 4 -

tissues which brings to the appearance in these cells or tissues of traits which said cells or tissues did not possess beforehand or to modulation of traits present, *a priori*, in the plants. The term "*stable transformation*" will be used to denote such a genetic transformation which is heritable to future generations of the transformed plant. The term "*transforming DNA*" will be used herein to denote a foreign DNA molecule which is introduced into plant cells and causes their transformation. The transforming DNA may comprise coding sequences and/or control sequences capable of regulating the amount and time of the transcription.

In accordance with the present invention it was surprisingly found that there exist conditions which allow stable transformation of *Lemnaceae* plants. Thus, by one of its aspects, the present invention concerns a stably transformed *Lemnaceae* plant, tissues, products thereof and progeny thereof.

The *Lemnaceae* plants are preferably of the genera: *Spirodela*, *Lemna* and *Wolffia*. The present invention preferably concerns transformed *Lemnaceae* strains capable of exceptionally high efficiency of transformation, an example of such a strain being *Spirodela punctata* strain 8717, which is a *Spirodela punctata* strain isolated by E. Landolt and erroneously labeled as *Lemna disperma* in Landolt 1986.

The transformed *Lemnaceae* plant, tissue and products thereof of the invention may be used for the production of various chemical and biological products such as proteins and polypeptides encoded by the transforming DNA and may also be used to prepare various enzymes capable of producing various chemicals such as carbohydrates, lipids, alkaloids, pigments, vitamins, etc.

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The present invention is also concerned, by another of its aspects, with chemical and biological products such as proteins, polypeptides, carbohydrates, lipids, alkaloids, pigments, vitamins, and others, obtained from the above stably transformed *Lemnaceae* plants.

The transformed plant or tissue may also express desired traits which are not featured in production of new products, examples of such

- 5 -

traits are: antibiotic resistance, for example, kanamycin resistance, conferred by the *npt II* gene; or herbicide resistance, for example, resistance to the herbicide BASTA 20 (ammonium glufosinate, Hoechst, Germany).

5 The transferred *Lemnaceae* plants or tissue may also express more than one foreign gene, for example, the plant may be transformed to be resistant to several herbicides and/or antibiotics at once.

In accordance with the present invention, it was found that stable transformation of *Lemnaceae* plants or tissue may be obtained by the use of *Agrobacterium* cells carrying said transforming DNA. Thus, in accordance  
10 with a further of its aspects, the present invention concerns a method for the stable transformation of *Lemnaceae* plants or tissue which comprises incubating *Lemnaceae* plants or plant tissue with *Agrobacterium* cells carrying said transforming DNA, whereby cells in said plant tissue become stably transformed by said transforming DNA.

15 It was further found that there exists *Agrobacterium* strains which can specifically target and transform meristematic tissue in *Lemnaceae*, for example *A. tumefaciens* strains EHA105, EHA101 and GVE3103, or *Agrobacterium* strains which can specifically target and transform the wounded area of the plant such as *A. tumefaciens* strains LBA4404 and C58.  
20 Therefore the method of the invention preferably concerns incubation of *Lemnaceae* plants with *Agrobacterium* of the strains. EHA105, EHA101 and GVE3103 capable of transforming the meristematic tissue or *Agrobacterium* strains LBA4404 and C58 capable of transforming wounded tissue.

25 It was still further found that use of vacuum filtration during the incubation of the *Lemnaceae* plants with the *Agrobacterium* cells increases the efficiency of transformation. Thus, by a preferred embodiment, the method of transformation includes incubation of *Lemnaceae* plants or tissue with *Agrobacterium* cells while applying vacuum infiltration.

30 Another embodiment of the method of the invention is based on the finding that it was possible to increase the efficiency of *Lemnaceae*

- 6 -

transformation by *Agrobacterium* by exposing the meristematic zone of the mother frond. Such exposure can be carried out physically, i.e. by removing the daughter frond to expose the meristematic zone, for example, by a plucking motion using forceps, or by any other mechanical means.

5 Alternatively, said exposure may be carried out by applying chemical preparation or a hormone preparation capable of specifically removing the daughter frond without damaging the underlying meristematic zone.

Yet another aspect of the present invention concerns a novel method for plant transformation using transforming *Agrobacterium* cells, which is particularly suitable for mass transformation of plant tissue. In accordance with this method, plants are cut into small particles which are then incubated with the transforming *Agrobacterium* cells, preferably in the presence of the booster medium which will be described hereinbelow. The size of the particles should be such that at least some of them will contain undamaged meristematic tissue which is capable of regenerating into full plants. In order to achieve this feature, the particles should preferably be at an average size of above 150  $\mu\text{m}$  in diameter, most preferably at a size range of about 150  $\mu\text{m}$  - 750  $\mu\text{m}$ . Cutting the plant tissue into such small particles maximizes the contact area between the meristematic tissue and the *Agrobacterium*. Furthermore, *Agrobacterium* cells more readily infect damaged plant tissue and by cutting the plant, the *Agrobacterium* cells are exposed to large regions of damaged plant tissue. The overall result of these factors is a marked increase in the transformation yield.

Another transformation method which may be used in the performance of the present invention, is microinjection which is known *per se*. In accordance with this method, *Agrobacterium* cells, preferably together with the booster medium of the invention which will be described hereinbelow, are microinjected to a desired zone of transformation within the plant, typically into the plant's meristem. One major advantage of microinjection, is that it allows specific targeting of the transforming *Agrobacterium* cells to a desired tissue, e.g. only to the roots' meristem, only to the leaves'

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meristem, etc., so that the result is a plant having foreign DNA only at a specific tissue, for example, the roots and not in other tissues.

Another embodiment of the method of transformation of *Lemnaceae* is based on the surprising finding that transformation can be carried out *in planta*, i.e. utilizing the full plant and there is no need to cut the plant to small particles, or to use tissue culture and then *in vitro* regeneration for transformation purposes. A full plant can be used for transformation provided that the *Agrobacterium* cells are targeted to the meristem either by direct microinjection as described above or by utilization of *Agrobacterium* strains which preferably target the meristem such as *A. tumefaciens* strain EHA105, EHA101 and GVE3103. Thus the present invention provides a method for *in planta* transformation of *Lemnaceae* by targeting *Agrobacterium* cells carrying the transforming DNA to the meristem of the plant to be transformed.

In accordance with another embodiment of the invention, it was found that it is possible to increase the efficiency of transformation of plants by *Agrobacterium* cells by incubating the *Agrobacterium* cells with the plant tissue to be transformed in the presence of a booster medium which is capable of increasing the *Agrobacterium's* virulence. This increase in efficiency due to use of the booster is not limited to the transformation of *Lemnaceae* plants but is also applicable to plants in general including monocotyledonous plants and dicotyledonous plants.

*Agrobacterium* is already routinely used for transformation of dicotyledonous plants. However, in accordance with this embodiment of the invention, the efficiency of transformation of dicotyledonous plants is increased by incubation of the *Agrobacterium* booster medium. With respect to monocotyledonous plants, although there have been some reports of a few successful transformations of such plants by *Agrobacterium*, these reports have been sporadic and usually showed unsatisfactory transformation yields. Increasing *Agrobacterium's* virulence by the booster medium, in accordance with said embodiment, allows for the first time, the transformation of many

species of monocotyledonous plants which were previously untransformed, including those belonging to the genus *Lemnaceae*, as well as an increase in the yield of transformation of plants already known to be transformed, albeit at a low yield by the use of *Agrobacterium*.

5           Stably transformed plants produced by utilizing the booster medium as described above, also form an aspect of the invention.

          The booster medium which enhances the virulence of the *Agrobacterium* cells, comprises plant tissue cultured at a pH below about 5.2. For example, the booster medium may comprise a fresh cell  
10       suspension of dicotyledonous plants, at a concentration of 1-10% (w/v). The fresh cell suspension may be, for example, from dicotyledonous plants of the *Solanaceae* family.

          Preferably, the booster medium also comprises caffeine at a concentration of 100-500 mg per liter of medium.

15           A specific example of a booster medium is one comprising MS basal medium at a pH of about 3.5 - 4.2, 1-10% (w/v) of a fresh cell suspension of *Nicotiana tabacum*, and about 100-500 mg per liter of medium caffeine.

          By another alternative, the booster medium of the invention is a  
20       plant growth medium comprising *Lemnaceae* plant extracts. Such a medium can be produced by extracting *Lemnaceae* plants in a suitable medium such as phosphate buffer.

          Both types of booster mediums, having either or both of the above specifications for use in enhancing transformation efficiency of  
25       *Agrobacterium* cells used as a transformation vector, also form another aspect of the invention.

          By yet a further embodiment, the present invention concerns a method for maintaining morphogenetic *Lemnaceae* calli for long periods of time, by using low levels of sucrose in the growth medium, for example,  
30       from 0.1 to 1.5% sucrose. It was found that it is possible to increase significantly the period of maintaining calli in a viable state, for example,

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from 2 weeks to more than 3 months by decreasing the sucrose level in the growth medium.

Another aspect of the invention concerns a method for the production of highly regenerative *Lemnaceae* calli, and furthermore a method for rapid and efficient regeneration of the plants from the calli, utilizing the combined effect of B5 minerals, low sucrose levels (0.1 to 1.5% sucrose) and phytohormones in the growth medium.

The present invention will now be illustrated with reference to some non-limiting examples.

#### BRIEF DESCRIPTION OF THE DRAWING

Fig. 1 shows a schematic picture of plasmid Ti pME504.

#### DETAILED DESCRIPTION OF THE INVENTION

In the following, the invention will be illustrated at times with specific reference to the transformation of *Lemnaceae* plants, tissue or callus, such reference being given merely as an example and it should be understood that the invention is not limited thereto.

The stably transformed *Lemnaceae* plants contain foreign genes which confer useful traits such as: improvement of nutritional quality of the plant or plant parts; *de novo* expression of desired chemical and biological products, e.g. enzymes; growth factors, hormones such as insulin; antibodies; anti-oxidants; defensins; proanthocyanidins; cytokines and other biologically active polypeptides and proteins; over-expression of products already expressed by these plants; etc. Other products that may be obtained from the transformed *Lemnaceae* plants are enzymes for industrial applications, such as super-oxide dismutase (SOD),  $\alpha$ -amylase, invertase, sucrose phosphate synthase and the like, and chemicals such as food pigments, e.g.  $\beta$ -carotene, anthocyanin, etc. The type of product obtainable from the transformed plants is obviously contingent on the nature of said transforming DNA.

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The transforming DNA which is introduced into the plant cells, include, as will be appreciated by the artisan, coding sequences which code for the desired trait as well as control sequences which control expression of the coding sequence, for example, promoters, enhancers and the like.

5 Furthermore, the transforming DNA may comprise also a selectable marker gene, such as a gene coding for herbicidal resistance, resistance to antibiotics, or the like. In addition or in the alternative, the transforming DNA may further comprise a reporter gene, such as a gene coding for a color marker. A selectable marker gene or a reporter gene facilitates identification and  
10 selection of the transformed tissue and enables its separation from untransformed tissue.

Specific examples of selectable marker genes are the hygromycin phosphotransferase (HPT) coding sequence, which may be derived from *E. coli* and which confers resistance to the antibiotic hygromycin B; the  
15 aminoglycoside phospho-transferase gene of transposon Tn5 (AphII) which encodes resistance to the antibiotics kanamycin; neomycin and G418; genes which code for resistance or tolerance to glyphosate, 2,2-dichloropropionic acid, methotrexate, imidazolinone herbicides, sulfonylurea herbicides, bromoxynil, phosphinothricin and other herbicidal compounds; and many  
20 others. Those selectable marker genes which confer resistance or tolerance to these phytotoxic compounds are also of commercial utility in the resulting transformed plants.

Reporter genes may be used for identifying transformed cells, tissue or calli and for evaluating the functionality of regulatory sequences.  
25 Reporter genes which code for easily assayable selectable marker proteins are well known in the art. In general, a reporter gene is a gene which is not present in, or expressed by, the recipient organism or tissue and which codes a protein which expression is manifested by some easily detectable property, e.g., phenotypic change or enzymatic activity. Examples of such genes are  
30 the chloramphenicol acetyl transferase gene (*CAT*) from Tn9 of *E. coli*, the  $\beta$ -glucuronidase gene (*GUS*) of the *uidA* locus of *E. coli*, the green fluorescence protein (GFP) obtained from *A. Victoria* and the luciferase



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gene from the firefly *Photinus pyralis*. Expression of the reporter gene is assayed at a suitable time after the DNA has been introduced into the recipient cells. An example of such an assay entails the use of the *E. coli*  $\beta$ -glucuronidase (*GUS*) gene (Jefferson *et al.*, (1987)). Plant cells transformed and expressing this gene will stain blue upon exposure to the substrate, 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronide (X-GLUC), in the extracellular medium.

According to the method of the invention, *Agrobacterium*, e.g. *Agrobacterium tumefaciens*, is engineered so as to contain the transforming DNA to be inserted into the target plant, e.g. the target *Lemnaceae* plant, which engineering is performed by means known *per se*. The whole plant or the plant cells tissue or callus are then brought into contact with the *Agrobacterium* cells and incubated together. The plant tissues are then selected for those containing the transforming DNA, for example, by testing for phenotypic expression of the marker gene, e.g. herbicidal or antibiotic resistance, or for the expression of the reporter gene, e.g. a color product. It is also possible to verify the presence of the introduced transforming DNA by a DNA assay such as by PCR.

The invention will now be illustrated further in the following examples:

## EXPERIMENTAL PROCEDURES

### I. Culture and maintenance of *Lemna* and *Spirodela* for micro-injection experiments

For meristem-zone microinjection, an axenic inoculum (approx. 10 plants) of *Spirodela oligorrhiza* (herein called *Spirodela punctata*) Hegelm. or *Lemna gibba* Hurfeish was introduced in a 250 ml flask containing 50 ml of MS medium having the ingredients as detailed in the following Table 1.

### II. Standard growth conditions

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Cultures were grown at 26°C under continuous fluorescent light ( $30 \mu\text{E m}^2\text{s}^{-1}$ ) in a 3-5%  $\text{CO}_2$ -enriched atmosphere.

Table 1:  
Modified MS basal medium (MS medium) (based on Murashige  
& Skoog, 1962)

Macro elements	Amount (mg/l)	Micro elements	Amount (mg/l)	Organic additives	Amount (mg/l)
$\text{NH}_4\text{NO}_3$	1650	$\text{H}_3\text{BO}_3$	6.2	Glycine	2
$\text{KNO}_3$	1900	$\text{MnSO}_4$	22.3	Meso-inositol	100
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	440	$\text{ZnSO}_4 \cdot 2\text{H}_2\text{O}$	0.25	Thiamine HCl	10
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	370	KI	0.83	Nicotinic acid	0.5
FeEDTA	35	$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.25	Pyridoxine	0.5
$\text{KH}_2\text{PO}_4$	170	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.25	Biotin	0.5
		$\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$	0.03	Folic acid	0.5
				Casein hydrolysate	800
				Sucrose	30000
				pH brought to 5.8 with NaOH prior to autoclaving	

### III. Standard Transformation Procedure

Under sterile conditions, 2.5 g *Lemnaceae* plants were placed in an empty 9 cm Petri dish. A 10 ml suspension of  $5-8 \times 10^8/\text{ml}$  *A. tumefaciens*, in MS Basal medium (Table 1) was prepared as described in (IV) below, and added to the dish. Plants were co-cultivated with the *A. tumefaciens* suspension for 20-40 min. at room temperature. The suspension was removed from the dish and the plants washed 3 times with fresh MS Basal medium (Table 1). The plants were transferred to a vessel (15x20x12cm) containing 1.5 l SP medium (Table 2) at 26°C under continuous fluorescent light ( $30 \mu\text{E m}^2\text{s}^{-1}$ ) in a 3-5%  $\text{CO}_2$ -enriched atmosphere.

Table 2

SP medium (modified from Hutner) (cf., Posner, 1967)

	Amount (mg/l)
KNO <sub>3</sub>	300
Ca(NO <sub>3</sub> ) <sub>2</sub> ·4H <sub>2</sub> O	72
MgSO <sub>4</sub> ·7H <sub>2</sub> O	74
KH <sub>2</sub> PO <sub>4</sub>	40
NaEDTA	0.003
Ferric citrate	1
H <sub>3</sub> BO <sub>3</sub>	1
MnSO <sub>4</sub>	0.1
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.1
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.03
ZnSO <sub>4</sub> ·4H <sub>2</sub> O	1
pH brought to 5.8 with NaOH prior to autoclaving	

#### IV. Preparation of *A. tumefaciens* for *Lemnaceae* transformation

- A single colony of *Agrobacterium tumefaciens*, maintained on antibiotic-supplemented LB plates (Suppl. LB medium;), (Table 3, below), was picked and grown overnight (28°C, 250 rpm) in 10 ml of antibiotic-supplemented 2YT broth (Suppl. 2YT 1 medium;) (Table 4 below). The grown culture was transferred to 50 ml of Suppl. 2YT medium and further grown for an additional 12 hr (28°C, 250 rpm). Before transformation, the *A. tumefaciens* culture was centrifuge (3200 x g, 5 min.), the supernatant discarded and the bacteria resuspended in 10 ml of MS medium (Table 1).
- Before co-cultivation with *Lemnaceae* plants, the bacterial concentration was adjusted to 5-8x10<sup>8</sup> cells/ml.

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Table 3

**Supplemented and solidified liquid broth medium  
(Suppl. LB medium)**

	Amount
Bacto tryptone	10 g
Bacto yeast extract	5 g
NaCl	10 g
Rifampicin	25 mg
Kanamycin	50 mg
Carbenicillin	50 mg
Cifco agar	10 g
pH brought to 7.0 with NaOH	

#### V. Meristem-zone microinjection

1. Preparation of *Lemnaceae* plants - Axenic *Lemnaceae* plants were cultured in containers (8.5 cm diameter by 11 cm height) containing 50 ml of MS medium (Table 1) at 25°C under cool white fluorescent bulbs (60  $\mu\text{E m}^{-2}\text{s}^{-1}$ ). All treatments were performed in a laminar-flow sterile cabinet at room temperature.
2. Preparation of the transformation vector - *Agrobacterium tumefaciens* containing a p35S GUS INT plasmid (Vancanneyt *et al.* 1990) was utilized. This plasmid carries the NPTII gene coding for kanamycin resistance and the coding sequence of the  $\beta$ -glucuronidase (GUS) *uidA* reporter gene (Jefferson, 1987) interrupted by the IV2 intron (Eches *et al.* 1986). Use of this vector enabled staining for GUS expression immediately after transformation and, at the same time, avoided *Agrobacterial*-derived, GUS-positive background. For transformation experiments, a single colony was picked and resuspended in Suppl. 2YT medium, the ingredients of which are detailed in the following Table 4:

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Table 4

**Supplemented 2YT liquid medium (Suppl. 2YT medium)**

	Amount/liter
Bacto tryptone	16 g
Bacto yeast extract	10 g
NaCL	5 g
Rifampicin	25 mg
Kanamycin	50 mg
Carbenicillin	50 mg
pH brought to 7.0 with NaOH	

The bacteria were cultured for 16 hours on a gyratory shaker (250 rpm) at 28°C. Before co-cultivating the bacteria and the plants, the bacterial culture was diluted with MS medium (Table 1) or booster medium (Table 5, below) to an optical density at 550 nm of 0.6 and brought to pH 4 with HCl. This yielded an *Agrobacterium* preparation suitable for transformation of *Lemnaceae*.

3. Microinjection - *Lemnaceae* plants were transferred to MS medium brought to pH 4, or to a booster medium of the invention having the ingredients as detailed in the following Table 5:

Table 5

***Agrobacterium* virulence-booster medium of the invention**

Component	Amount
Caffeine (Sigma)	150 mg
Fresh cell suspension from <i>Nicotiana tabacum</i> (2SH) (prepared as in Aviv and Galun, 1984)	20 ml
MS basal medium (Table 1) pH brought to 4.0 with HCl prior to autoclaving	980 ml

Plants were microinjected under a dissecting microscope using a 1 ml sterile disposable syringe with a G-27 needle, and filled with a preparation of *Agrobacterium* suitable for transformation of *Lemnaceae*.

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The injections were targeted toward the meristematic zones of the plants in order to bring the bacteria into close contact with the growing meristem. In each injection, about 20  $\mu$ l were injected inside the meristematic zone and each zone was injected three times.

- 5 4. Co-cultivation - The injected *Lemna* or *Spirodela* plants were co-incubated with the suitably prepared *Agrobacterium* in MS medium (Table 1) brought to pH 4, or in the booster medium of the invention (Table 5), for 48 hours at 25°C under cool white fluorescent bulbs (30  $\mu$ E m<sup>-2</sup>s<sup>-1</sup>). Following this, the plants were washed 3 times with sterile distilled water  
10 at room temperature and cultured in MS medium (Table 1) supplemented with 400 mg/l claforan.

#### VI. Standard X-Gluc Staining Procedure

- In an 1.8 ml Eppendorf tube, 5 mg of x-gluc (Duchefa  
15 Biochemie BV) were dissolved in 150  $\mu$ l of dimethyl formamide. Then the following staining solution was added: 10 ml of 100 mM Tris 7.0; 15  $\mu$ l of 500 mM ferrocyanide (stock kept frozen); 15  $\mu$ l of 500 mM ferricyanide (stock kept frozen) and 100  $\mu$ l of 10% Triton x-100. The plants were then transferred to a 9 cm Petri dish and 10 ml of staining solution were added.  
20 Tubes were incubated overnight at 37°C in darkness. Then the staining solution was discarded and rinsed with distilled water. The GUS positive plants were observed with a binocular microscope.

#### 25 VII. Callus formation and long-term maintenance of morphogenetic *Spirodela*

- 
- Spirodela punctata* plants were transferred to SP medium  
(Table 2) for 5 days at 26°C under continuous fluorescent light (30  $\mu$ E  
m<sup>-2</sup>s<sup>-1</sup>). The plants were placed under a binocular microscope, illuminated  
30 from below, and the growing daughter fronds removed, by a plucking motion using a forceps. For callus induction, mother fronds were grown on B-5 medium (Table 6, below) supplemented with 1.0% sucrose, 2 mg/l BA and 50 mg/l of Dicamba. After growing for 3 weeks on this medium, the

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calli were transferred to B-5 medium supplemented with 2 mg/l 2IP and 10 mg/l 2,4-D. Long-term maintenance of calli was achieved by periodical transfer every 4 weeks to fresh B-5 medium supplemented with 1.0% sucrose, 2 mg/l 2IP and 10 mg/l 2,4-D.

5

#### VIII. Rapid regeneration of *Spirodela* plants from calli

*Spirodela* calli were maintained on B-5 medium (Table 6 below) supplemented with 1.0% sucrose, 3 mg/l 2IP and 10 mg/l 2,4-D. For regeneration, calli were transferred to B-5 medium supplemented with 1.0% sucrose and 2 mg/l 2IP. Fully regenerated *S. punctata* plants were efficiently obtained within 1-2 weeks. *Spirodela* calli and the regenerated plants, were grown at 26°C under continuous fluorescent light ( $30 \mu\text{E m}^{-2}\text{s}^{-1}$ ).

10

Table 6

B-5 medium (modified from Gamborg *et al.*, 1968)

	Amount (mg/l)
KNO <sub>3</sub>	2500
MgSO <sub>4</sub> ·7H <sub>2</sub> O	250
Na <sub>2</sub> H <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O	150
CaCl <sub>2</sub> ·2H <sub>2</sub> O	150
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	134
FeEDTA	28
H <sub>3</sub> BO <sub>3</sub>	3
MnSO <sub>4</sub> ·H <sub>2</sub> O	10
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	2
Na <sub>2</sub> MoO <sub>4</sub> ·H <sub>2</sub> O	0.25
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.025
CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.025
KI	0.75
Nicotinic acid	1
Thiamine HCl	10
Pyridoxine HCl	1
<i>m</i> -inositol	100
Gelrite	3 g/l
pH brought to 5.8 with NaOH prior to autoclaving	

## IX. Semi-automated meristem isolation and transformation

1. Synchronization of *Spirodela* and *Lemna* growth by meristemexposure - All materials were sterilized and all procedures were carried out

- under aseptic conditions. Plants (10 g) were harvested in a laminar-flow
- 5 sterile cabinet by pouring the contents of a culture vessel through a sterilized 10 mesh (1.7 mm pore size) stainless steel sieve. The *Spirodela* or *Lemna* plants were transferred from the top of the sieve with a sterile spoon to a 1 liter-capacity sterile blender, modified to contain 6 razor blades positioned in three different planes (Blumenthal *et al.*, 1993). The blender was filled



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with 250 ml of distilled, filter-sterilized water and activated for 4 sec. at 17000 rpm. The partially homogenized plants were poured aseptically from the blender through a 20 mesh (800  $\mu$  pore size) sterilized Nitex sieve and collected on a 42 mesh (350  $\mu$  pore size) sterilized Nitex sieve. Subsequently, a jet of filter-sterilized distilled water (2 atm., 10 liters/min) was directed on top of the 20 mesh Nitex sieve in order to force all of the explant particles smaller than 800  $\mu$ m to pass through, yielding a particle size population of 350–750  $\mu$ m. Explant particles larger than 800  $\mu$ m were transferred back to the blender with a sterile spoon, the blender activated for 3 sec at 17000 rpm, and all sieving and washing steps repeated as above. The combined size population of 350–750  $\mu$ m, collected on a 42 mesh sterilized Nitex sieve, was subjected to a final sterile water-jet wash. The 350–750  $\mu$ m sieved explant particles (5 g) were collected by sterile spoon and spaced in a 14 cm diameter sterile petri dish. Throughout this process, all the waste was directed by gravitational force to a 30 liter plastic container situated below the laminar flow cabinet.

2. Co-cultivation followed by recovery period - All materials were sterilized and all procedures were carried out under aseptic conditions. The sieved 350–750  $\mu$ m explant particles from *Spirodela* or *Lemna*, in the 14 cm diameter petri dish, were resuspended in 15 ml of MS medium (Table 1) brought to pH 4, and then mixed with 15 ml of *Agrobacterium* previously cultured in Suppl. 2YT medium (Table 4) for 24 hrs. at 25°C on a gyratory shaker at 250 rpm. The explant-bacteria mixture was co-cultivated for 1 hr at 25°C and 10  $\mu$ E m<sup>-2</sup>s<sup>-1</sup>. Following this, the mixture was transferred by spoon to a 100 mesh (150  $\mu$ m pore size) sterilized Nitex sieve. The particles excluded by the sieve were washed with 5 ml of MS medium (Table 1) brought to pH 4, and transferred to a 3 liter wide-mouth culture vessel containing 500 ml of the same medium. Co-cultivation continued for 48 hr. at 25°C under continuous fluorescent light (30  $\mu$ E m<sup>-2</sup>s<sup>-1</sup>). At the end of this period, the mixture was sieved through a 100 mesh (150  $\mu$ m pore size) Nitex sieve, followed by three 200 ml rinses with distilled water. Explant particles with associated bacteria were transferred by spoon to a 3 liter

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wide-mouth culture vessel containing 500 ml of MS medium (Table 1) supplemented with 400 mg/l claforan and cultured for 5 days at 25°C and 30  $\mu\text{E m}^{-2}\text{s}^{-1}$  light. The explants were then sieved as before, followed by separation of the floating material (most of which was living and proliferating) from the sunken, non-vital particles. This was achieved by pouring the explant material together with distilled water, into a 200 ml graduated cylinder and collecting the floating material by a straining spoon. Explants were cultured in 1 liter Erlenmeyer flasks containing 300 ml of SP medium (Table 2) for 3 days.

## X. Selection and reporter genes

NPT II Selection and GUS staining were used to determine whether the cells of the treated plants tissue or calli were transformed, i.e. that they contained the transforming DNA. A DNA comprising the caMV 35s promoter was used followed by the *E. coli* NPT II coding sequence, which confers kanamycin resistance, as an expression vector. Stable inheritance of transgenic traits (kanamycin resistance and GUS activity) were assayed on permissive media while the NPT II gene and its products were assayed by PCR and immuno-blotting, respectively.

### Example 1:

#### Transformation of *Lemna* and *Spirodela* plants by microinjection

A color-marker reporter gene (*GUS*) and a gene conferring resistance to the antibiotic kanamycin (*NPT II*) were transferred into the *Lemnaceae* plants (*Lemna gibba* Hurfeish and *Spirodela punctata*) by *Agrobacterium tumefaciens* mediated transformation. This was achieved after suitably preparing the plants and actively promoting DNA transfer into the plant nucleus as specified above.

Booster medium of the invention (Table 5) markedly enhanced *Agrobacterium* virulence against *Lemnaceae*. Applying *Agrobacterium* to *Lemna* or *Spirodela* plants, maintained for two months in MS medium (Table 1), while omitting the booster medium drastically reduced micro-

injection-mediated transformation frequencies as shown in the results of the following experiments:

**Experiment 1.1:**

5           49 out of 100 microinjected *Lemna* plants maintained in MS medium were GUS positive when the booster medium of the invention was used, while 3 out of 100 were GUS positive when the booster medium was omitted.

10   **Experiment 1.2:**

          47 out of 100 microinjected *Spirodela* plants maintained in MS medium were GUS positive when the booster medium of the invention was used, while 2 out of 100 were GUS positive when the booster medium was omitted.

15           Experiments 1.1 and 1.2 thus prove that the booster medium of the invention significantly raises the efficiency of transformation.

**Experiment 1.3:**

20           34 out of 100 microinjected *Lemna* plants maintained in MS medium were GUS positive when the booster medium of the invention was brought to pH 4.0; 19 out of 100 were GUS positive when the booster medium was brought to pH 5.2; and 9 out of 100 were GUS positive when the booster medium was brought to pH 7.5.

25   **Experiment 1.4:**

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          31 out of 100 microinjected *Spirodela* plants maintained in MS medium were GUS positive when the booster medium of the invention was brought to pH 4.0; 13 out of 100 were GUS positive when the booster medium was brought to pH 5.2; and 5 out of 100 were GUS positive when  
30   the booster medium was brought to pH 7.5.

          These results clearly indicate that a pH below about 5.2 raises the efficiency of transformation.

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The addition of caffeine, a novel agent in transformation protocols, and live tobacco cells (Aviv and Galun, 1984), was found to promote *Agrobacterium* transformation, as shown in the following experiments:

5

**Experiment 1.5:**

44 out of 100 microinjected *Lemna* plants maintained in MS medium were GUS positive when co-cultivation was carried out in MS medium brought to pH 4.0, which contained caffeine and live tobacco cells; while only 33 out of 100 were GUS positive when caffeine and tobacco cells were omitted.

10

**Experiment 1.6:**

39 out of 100 microinjected *Spirodela* plants maintained in MS medium were GUS positive when co-cultivation was carried out in MS medium brought to pH 4.0, which contained caffeine and live tobacco cells; while only 31 out of 100 were GUS positive when caffeine and tobacco cells were omitted.

15

Experiments 1.5 and 1.6 thus indicate that addition of caffeine and live tobacco cells to the booster medium of the invention raises the transformation efficiency.

20

**Example 2: Development of plants from explant particles**

The semi-automated *Lemnaceae* blending process resulted in a purified fraction of explant particles 350–750  $\mu\text{m}$  in size, from either *Lemna* or *Spirodela*, which represented approximately 50% of the total starting material. Among the 350–750  $\mu\text{m}$  particles were explant particles which were seen to contain undamaged meristematic zones, from which new plants vigorously grew. Explant particles smaller than 150–350  $\mu\text{m}$  gave drastically reduced number of actively growing plants. The meristem-containing explants remained green, floated and grew to maturity. All other explant particles rapidly turned yellowish-brown, eventually bleaching entirely, and

25

30

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most sunk to the bottom of the culture vessel. The massive death of the non-meristematic explants sections did not inhibit the normal development of new plants, which developed from the meristem-containing explant sections into mature *Lemnaceae* plants morphologically indistinguishable from non-blended plants, as demonstrated in the following experiments:

#### Experiment 2.1:

Forty-eight hours after the blending process, 600-800 explant particles out of a total of 80,000 gave rise to tiny green *Spirodela* plants. The incipient colonies each contained 1-2 plants not longer than 1 mm. Six days after blending, these colonies consisted of 3 plants, each 2-3 mm long, and 2-3 newly developed roots (5-6 mm in length). Nine days after blending, the plants reached an average size of about 4-5 mm long and a shape both comparable to that of non-blended control *Spirodela*. At this stage, colonies contained 5-7 fronds and 5-6 fully elongated roots. These plants were further subcultured for at least 5 generations. The average biomass doubling time was 2 days and was not distinguishable from that of control plants. No somaclonal variation was observed.

#### Experiment 2.2:

Forty-eight hours after the blending process, 300-500 explants particles out of a total of 80,000 gave rise to tiny green *Lemna* plants. The incipient colonies each contained 1 plant not longer than 1 mm. Six days after blending, these colonies consisted of 2 plants, each 2-3 mm long, and 2 newly developed roots (1 mm in length). Nine days after blending, these new plants reached the average size of about 7 mm long and had a shape comparable to that of non-blended control *Lemna*. At this stage, colonies contained 4 plants and 2-4 roots. These plants were further subcultured for at least 5 generations. The average biomass doubling time was 2 days and was not distinguishable from that of control plants. No somaclonal variation was observed.

**Example 3:**

**Analysis of *Lemna* and *Spirodela* plants transformed by the semi-automated meristem-Lemnaceae-blending process and by the transformation procedure**

5

**A. GUS positive staining in transformed *Lemnaceae* plants following semi-automated meristem exposure and transformation.**

Following transformation with *Agrobacterium* harboring the  $\beta$ -glucuronidase (GUS) *uidA* reporter gene, explants were cultured for 3-7 days and then stained for GUS activity. The following are results of two such experiments:

10

**Experiment 3.1:**

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Sixteen hours after immersing *Agrobacterium*-transformed *Spirodela* colonies in a GUS reaction mixture (Jefferson, 1987), 120 of 600 colonies in one repetition, and 400 of 800 colonies in another, exhibited blue sectors, indicating that 20-50% of the explants were transformed. The size of transformed sectors ranged from 0.01 to 1 mm<sup>2</sup>. Untransformed control plants did not exhibit any GUS staining. In 2 out of the 600 and 16 of the 800 colonies (0.3-2%), daughter generation plants were stained systematically blue. This indicated that the meristematic zones from which the daughter plants regenerated, had been transformed. In some colonies, the mother generation plant remained unstained while systemic GUS staining was observed in some of the daughter generation plants. This indicates that mass meristem exposure of *Spirodela* plants can lead to meristem-targeted transformation.

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25

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**Experiment 3.2:**

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Sixteen hours after immersing *Agrobacterium*-transformed *Lemna* colonies in a GUS reaction mixture (Jefferson, 1987), 60 of 300 colonies in one repetition, and 250 of 500 colonies in another, exhibited blue sectors, indicating that 20-50% of the explants were transformed. The size of transformed sectors ranged from 0.01 to 1 mm<sup>2</sup>. Untransformed control

plants did not exhibit any GUS staining. In 1 out of the 300 and 5 of the 500 colonies (0.3–1%), daughter generation plants were stained systemically blue.

## 5 B. Integration of the kanamycin resistance gene

In order to verify insertion and integration of the transferring DNA from *Agrobacterium*, total DNA was extracted from *Lemna* and *Spirodela* plants that were previously selected for positive GUS staining. The DNA was amplified in a PCR reaction (annealing at 55°C) with the  
10 following primers of the NPT II coding regions:

1. 5' GCACGAGGTTCTCCGGCCGCTTGGG 3';
2. 5' GAAGGCGATGCGCTGCGAATCGGG 3'.

These primers produce a 780 bp fragment within the NPT II gene. The PCR reaction product were electrophoresed on an agarose gel (0.8%)  
15 and stained with ethidium bromide. GUS-positive *Lemna* and *Spirodela* plants exhibited the expected band at the expected size for the NPTII transgene. Untransformed controls of *Lemna* or *Spirodela* plants did not contain this band. The same band at the same migration position was evident also in DNA isolated from the Ti plasmid of *Agrobacterium*, which  
20 was electrophoresised on the same gel as a positive control. These results verified that *Agrobacterium* is capable of genetically transforming *Lemna* and *Spirodela* plants.

## 25 C. Transformation of specific organs in an intact *Lemnaceae* plant

In 5% of the transformed population, expression of the introduced GUS gene was detected only in the root system. In these cases, GUS  
expression was detected all over the root system. This is of importance in cases where it is of interest to express the introduced gene in only a defined part of the plant such as root tissue.

**Example 4 Identification of *Agrobacterium* strains which have a specificity towards transformation of meristemic tissue in *Lemnaceae***

5           *Spirodela punctata* and *Lemna gibba* var. Hurfeish plants were maintained in SP medium (Table 2) under standard growth conditions (Procedure II). Using the standard transformation procedure (Procedure III), intact plants were co-cultivated with 5 different *A. tumefaciens* strains (EHA105 [Xiu-Qing Li *et al.*, 1992]; EHA101 [Hood *et al.*, 1987];  
10 GVE3103 [Deblaere *et al.*, 1985]; LBA4404 [Ooms *et al.*, 1982]; and C58 [Van Larebeke *et al.*, 1974]) each harboring Ti plasmid pME504 (shown in Fig. 1). This plasmid carries: the *nptII* gene, conferring resistance to the antibiotic kanamycin, under the control of the nopaline synthase promoter; the *bar* gene, conferring resistance to the herbicide BASTA (Thompson *et al.*, 1987), under the control of the 35S-CaMV promoter; and the *uidA* gene  
15 interrupted by an intron (Vancanneyt *et al.*, 1990), coding for the GUS reporter, also under the control of the 35S-CaMV promoter. GUS expression (Procedure VI) was determined by scoring blue spots. The tissue specificity of the different *A. tumefaciens* strains was determined by scoring  
20 the distribution of blue spots in the *Lemnaceae* plants. The data are summarized in Table 7, below.

The method involved wounding *Spirodela punctata* and *Lemna gibba* Hurfeish plants, co-cultivating the plants with  $5 \times 10^8$  bacteria  $\text{ml}^{-1}$ , vacuum infiltration (30 mbar, 5-10 min) and further co-cultivation for 4 hr.  
25 Fronds were assayed for GUS expression 10 days after co-cultivation.

The results indicate that GUS expression in *S. punctata* co-cultivated with *A. tumefaciens* strains EHA105, EHA101 and GVE3103 was  
~~restricted mainly to daughter fronds arising from meristematic tissue, while~~  
GUS expression in *S. punctata* co-cultivated with *A. tumefaciens* strains  
30 LBA4404 and C58 was restricted mainly to wounded areas of the mother frond.



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Table 7

<i>A. tumefac.</i>	Ti plasmid	GUS expression (% of fronds)			
		Spirodela		Lemna	
strain	type	mother frond	daughter frond	mother frond	daughter frond
EHA105	agropine	6	23	1	7
EHA101	agropine	7	22	1	5
CVE3-	octopine	3	18	0	0
101	octopine	10	3	0	0
LBA4-	none	8	1	0	0
404					
C58					

**Example 5: Identification of *Agrobacterium* strains which specifically target and transform wounded tissue in *Lemnaceae***

*Spirodela punctata* and *Lemna gibba* Hurfeish were maintained in SP medium (Table 2) under standard growth conditions (Procedure II). Using standard transformation procedure (Procedure III), intact plants were co-cultivated with 5 different *A. tumefaciens* strains each harboring the Ti plasmid pME504 (shown in Fig. 1). GUS expression was determined by scoring blue spots. The tissue specificity of the different *A. tumefaciens* strains was determined by the distribution of spots in the *Lemnaceae* plants. GUS expression in *S. punctata* co-cultivated with *A. tumefaciens* strains LBA4404 and C58 was restricted mainly to wounded areas of the mother frond, while GUS expression in *S. punctata* co-cultivated with *A. tumefaciens* strains EHA105, EHA101 and GVE3103 was restricted mainly to daughter fronds arising from meristematic tissue as shown in Table 7, above.

**Example 6 Use of vacuum infiltration for increasing efficiency of transformation of *Lemnaceae* by *Agrobacterium***

*Spirodela punctata* and *Lemna gibba* Hurfeish plants were maintained in SP medium (Table 2) under standard growth conditions

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(Procedure II). Intact plants were co-cultivated with *A. tumefaciens* strain EHA105 harboring Ti plasmid pME504 using the standard transformation procedure (Procedure III) with and without vacuum infiltration (30 mbar, 5-10 min). Transformation efficiency was determined by scoring GUS expression (blue spots). The data are shown in Table 8 below.

The method involved wounding *Spirodela punctata* var. Helgm and *Lemna gibba* var. Hurfeish plants, co-cultivating them with  $5 \times 10^8$  bacteria  $\text{ml}^{-1}$  (*A. tumefaciens* strain EHA105 harboring Ti plasmid pME504) and vacuum infiltration (30 mbar for 5-10 min). Control plants were wounded and co-cultured as above but without vacuum infiltration. Plants were assayed for GUS expression 10 days after co-cultivation.

The data shows an increase in transformation efficiency of 61% for *Spirodela* and 400% for *Lemna* following vacuum infiltration.

Table 8

GUS expression (% fronds)			
	Control	Vacuum infiltrated	% increase
<i>Spirodela</i>	18	29	61
<i>Lemna</i>	2	8	400

**Example 7: Method for increasing efficiency of *Lemnaceae* transformation by *Agrobacterium* by exposing the meristematic zones of the mother frond**

In order to partially expose the meristematic zones of *Lemnaceae* mother fronds to *Agrobacteria*, plants were placed under a binocular microscope, illuminated from below, and the growing daughter fronds removed; for example, by a plucking motion using a forceps. This procedure had no effect on the viability of the treated fronds. An experiment involving 500 *Spirodela punctata* plants, half of which had their meristematic zones exposed by daughter frond removal, resulted in an

increase of GUS expression in meristematic zones from 14% (not treated) to 23% (meristematic zone exposed).

5      **Example 8: Method for increasing efficiency of *Agrobacterium* transformation of *Lemnaceae* by direct dissection and exposure of mother frond meristematic zones**

Following removal of the daughter fronds from the meristematic pockets of the mother frond, the mother frond was longitudinally dissected  
10      under the binocular microscope in order to fully expose its meristematic zones. An experiment was performed in which the daughter fronds were removed from 500 *Spirodela punctata* plants. Following this, 250 of these plants were also longitudinally dissected. GUS expression was monitored 10 days following co-cultivation with *A. tumefaciens* EHA105 harboring Ti  
15      plasmid pME504. GUS expression was observed in 33% of the longitudinally dissected plants, compared with 25% in the non-dissected ones.

20      **Example 9: Method for increasing stability of *Agrobacterium* transformation of *Lemnaceae* by direct dissection and exposure of mother frond meristematic zones**

The method involved treating five hundred *Spirodela punctata* as described in Example 8. For all plants, daughter fronds were first removed. In addition, one half of the mother fronds were, longitudinally dissected and  
25      co-cultivated with  $5 \times 10^8$  bacteria  $\text{ml}^{-1}$  *A. tumefaciens* strain EHA105 harboring Ti plasmid pME504. Plants were vacuum infiltrated (30 mbar for 5-10 min). Plants were assayed for GUS expression 5, 10 and 15 days after co-cultivation and their filial relationship to the dissected mother frond was  
~~recorded. The results are shown in Table 9~~

- 30 -

Table 9

GUS expression (% fronds)						
Treatment	Mother frond	Daughter front generation				
	F <sub>0</sub>	F <sub>1</sub>	F <sub>2</sub>	F <sub>3</sub>	F <sub>4</sub>	F <sub>5</sub>
Longitudinally dissected	0	19	5	2	1	0.2
Non dissected	6	15	3	1	0	0.0

An increase in the stability of GUS expression meristematically transformed from one filial generation to the next was obtained as evident from Table 9.

5 **Example 10: Utilization of *Lemnaceae* extracts for increasing efficiency of transformation**

*Spirodela punctata* plants were maintained in SP medium (Table 2) under standard growth conditions (Procedure II). Using standard  
10 transformation procedure (Procedure III), plants were co-cultivated with *A. tumefaciens* strain EHA105 harboring Ti plasmid pME504. Plants were wounded or left intact (non-wounded). Thereafter, they were co-cultivated with *A. tumefaciens* in SP medium and supplemented for various periods of time with an extract from *Spirodela* plants. Transformation efficiency was  
15 determined by scoring GUS expression (blue spots).

The method involved maintaining 500 *Spirodela punctata* plants treated as described in Procedure III. Of these 500 plants, 250 were wounded. All plants were co-cultivated with  $5 \times 10^8$  bacteria ml<sup>-1</sup> (*A. tumefaciens* strain EHA105 harboring Ti plasmid pME504), and exposed to  
20 an extract from *Spirodela*. The extract was prepared by homogenizing 20 g of *Spirodela* plants from a one-week old culture in 50 ml phosphate buffer (pH 7.0). The homogenate was centrifuged (10 min. 10000 rpm) and the supernatant filter sterilized. The resulting *Spirodela* extract was applied for 4 hr during co-cultivation, or for this period plus the subsequent 10

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days. Control plants were not exposed to the extract. All plants were assayed for GUS expression 10 days after co-cultivation.

The data summarized in Table 10 show that the presence of the *Spirodela* extract enhanced the transformation efficiency of the non-wounded plants.

Table 10

Incubation with <i>Spirodela</i> extract (h)	GUS expression (% of fronds)	
	Wounded	Non-wounded
0	16	8
4	17	10
240	16	14

#### Example 11 Demonstration of transformability of several species from a number of genera of *Lemnaceae*

All plants were maintained in SP medium (Table 2) under standard growth conditions (Procedure II). Using standard transformation procedure (Procedure III), plants were co-cultivated with *A. tumefaciens* strains EHA105 harboring Ti plasmid pME504. Table 11 summarizes the percentage of plants from different *Lemnaceae* species expressing GUS 10 days after co-cultivation. Gus expression in F<sub>1</sub> daughter fronds was determined for three different genera of *Lemnaceae*: (A) *Spirodela* (B); (b) *Lemna*; and (C) *Wolffia*, all inoculated with *A. tumefaciens* strain EHA105 (pME504).

The method involved wounding plants, co-cultivating them with  $5 \times 10^8$  bacteria ml<sup>-1</sup> (*A. tumefaciens* strain EHA105 harboring Ti plasmid pME504), vacuum infiltration (30 mbar for 5-10 min.) and then further co-cultivation for 30 min. Plants were assayed for GUS expression (blue stain) 10 days after co-cultivation.

The results demonstrate the general applicability of the method of the invention for *Lemnaceae* transformation.

Table 11

Genus	Species	Strain	% stained plants
<i>Spirodela</i>	<i>intermedia</i>	7797	3
<i>Spirodela</i>	<i>punctata</i>	8717	92
<i>Spirodela</i>	<i>punctata</i>	Hegelm	27
<i>Lemna</i>	<i>obscura</i>	7325	12
<i>Lemna</i>	<i>obscura</i>	7780	14
<i>Lemna</i>	<i>gibba</i>	Hurfeish	8
<i>Lemna</i>	<i>gibba</i>	G-3	5
<i>Wolffia</i>	<i>brasiliensis</i>	8743	0.1
<i>Wolffia</i>	<i>australiana</i>	8730	9

**Example 12: Transformed *Lemnaceae* expressing antibiotic resistance**

*Spirodela punctata* plants obtained by standard transformation procedure (Procedure III) were placed in SP medium (Table 2) containing 2  $\mu\text{g/ml}$  kanamycin. In non-transformed control cultures, newly emerging plants grew white in the presence of the antibiotic. However, following transformation and culturing in the presence of kanamycin (2  $\mu\text{g/ml}$  for two months), three out of 500 (Experiment 1), and three out of 300 (Experiment 2) newly emerging plants were green and resistant to the bleaching effects of the antibiotic. This indicated that the *NPTII* gene was present and expressed in the green, resistant plants. The following plants were monitored: a kanamycin resistant clone 10 generations after the start of Experiment 2; a kanamycin sensitive colony (Kn-) with bleached daughter fronds, which did not develop further in the presence of kanamycin during the two months of the experiment; a non-transformed control showing a colony with bleached daughter fronds after 7 days exposure to 2  $\mu\text{g/ml}$  kanamycin. Staining of a sample taken from the kanamycin resistant clone two months after the start of Experiment 2 showed blue GUS staining in more than 80% of the fronds. The results are shown in Table 12.

The method involved wounding plants, co-cultivating them with  $5 \times 10^8$  bacteria  $\text{ml}^{-1}$  (*A. tumefaciens* strain EHA105 harboring Ti plasmid pME504) for 30 min. and vacuum infiltration (30 mbar, for 5-10 min).

Transformed *Spirodela punctata* plants were grown in SP medium (Table 2) supplemented with 2 µg/ml kanamycin for 2 to 5 weeks. Six green plants resistant to kanamycin, and a sampling of bleached ones, were assayed for GUS expression.

5 The data summarized in Table 12 indicate that the green, antibiotic-resistant plants were indeed transformed.

Table 12

Supplement to SP medium	GUS expression (% fronds)	
	Green plants	Bleached plants
Kanamycin (2 µg/ml)	83	0
BASTA (2 µg/ml)	76	0

**Example 13: Transformed *Lemnaceae* carrying herbicide resistance**

Five hundred plants of *Spirodela punctata* 8717 were co-cultivated with *A. tumefaciens* EHA105 (pME504), carrying: the *nptII* gene conferring resistance to the antibiotic kanamycin; the *bar* gene conferring resistance to the herbicide BASTA; and the *uid A* gene (interrupted by an intron (Vancanneyt *et al.*, 1990) coding for the GUS reporter. Co-cultivated plants were grown in SP medium (Table 2) supplemented with 2 µg/ml BASTA for 5 weeks. The plants were periodically transferred to fresh BASTA supplemented medium every 2 weeks. Under these conditions, control plants failed to grow and eventually bleached completely after 16 days. Thirty four out of 500 plants co-cultivated with *A. tumefaciens* EHA105 (pME504) were resistant to the bleaching effects of the herbicide.

This indicated that the *bar* gene was present and expressed in these green, growing plants. The following plants were monitored: green plants from a herbicide resistant clone; a BASTA sensitive, bleached plant (BASTA-) which failed to develop F<sub>1</sub> daughter fronds; and a control (non-transformed controlled showing a bleached plant. The plants were monitored 16 days after co-cultivation. Staining of a sample taken from the BASTA resistant

clone 25 days after the start of the experiment showed blue GUS staining in more than 75% of the fronds. The data is summarized in Table 12. The results indicate that the green, herbicide-resistant plants, repeatedly selected on fresh BASTA supplemented medium, were indeed transformed.

5

**Example 14: Transformed *Lemnaceae* carrying fluorescence reporter genes**

*Spirodela punctata* plants were maintained in SP medium (Table 2) under standard growth conditions (Procedure II). Using the standard transformation procedure (Procedure III), plants were co-cultivated with *A. tumefaciens* EHA105 (pME506) carrying the *nptII* gene conferring resistance to the antibiotic kanamycin; the *bar* gene conferring resistance to the herbicide BASTA; and the *luc* gene coding for the firefly luciferase reporter LUC. In parallel, other plants were co-cultivated with *A. tumefaciens* EHA105 (pME508) carrying the *nptII* gene conferring resistance to the antibiotic kanamycin; the *bar* gene conferring resistance to the herbicide BASTA; and a gene coding for the green fluorescence protein (GFP) of *Aequorea victoria*. Expression of the fluorescence reporter genes in the *Spirodela* plants was determined (Millar *et al.*, 1992; Chiu *et al.*, 1996) 10 days after co-cultivation. Expression of GFP was found throughout the frond, when viewed at a magnification of 200 times.

25

**Example 15: Expression of multiple foreign genes in one transformed *Lemnaceae* plant**

*S. punctata* plants were co-cultivated with *A. tumefaciens* EHA105 (pME504), carrying: the *nptII* gene conferring resistance to the antibiotic kanamycin; the *bar* gene conferring resistance to the herbicide BASTA; and the *uidA* gene (interrupted by an intron (Vancanneyt *et al.*, 1990) coding for the GUS reporter. Transformed plants were grown in SP medium (Table 2) in the presence of either 2 µg/ml kanamycin or 2 µg/ml BASTA for 2-5 weeks. Resistant green plants, as well as a sample of

30



bleached plants, were assayed for GUS expression. The results, summarized in Table 12, demonstrate a high correlation between antibiotic- or herbicide-resistant green plants, and GUS-expressing plants. This demonstrates co-expression of multiple genes in transformed *Lemnaceae*.

5

**Example 16: Identification of a high-efficiency-transformation strain of *Lemnaceae***

Experiments demonstrated a high frequency of GUS staining of a representative population of transformed *Spirodela punctata* 8717 four days after transformation. The transformation rate for this strain is > 90% using the standard transformation procedure (Procedure III).

10

**Example 17: Stable, non-chimeric transformation of *Lemnaceae***

15

*Spirodela punctata* 8717 plants were maintained in SP medium (Table 2) under standard growth conditions (Procedure II). Using the standard standard transformation procedure (Procedure III), plants were co-cultivated with *A. tumefaciens* EHA105 (pME504), carrying the *nptII* gene conferring resistance to the antibiotic kanamycin; the *bar* gene conferring resistance to the herbicide BASTA; and the *uidA* gene interrupted by an intron (Vancanneyt *et al.*, 1990) coding for the GUS reporter (Fig. 1). The expression of the GUS reporter gene in transformed plants was periodically determined by sampling the population at 4, 10 and 35 days after co-cultivation. Representative examples show more than 7 successive generations (attached by their stipes) of transformed plants expressing GUS throughout their tissues. This indicates stable, non-chimeric transformation of entire *Lemnaceae* plants over several generations and an extended period of time.

20

25

**Example 18: GUS expression in *Lemnaceae* plants transformed with a promoterless *uidA* gene, indicating integration of foreign DNA into the *Lemnaceae* chromosome**

5        *S. punctata* plants were co-cultivated with *A. tumefaciens* GV3103 (pVCGUS) (Koncz *et al.*, 1989) which contains a promoterless GUS construct (the *uidA* gene interrupted by an intron). Transformed plants, expressing GUS seven days after co-cultivation, were monitored. Promoterless GUS expression is only possible if the *uidA* gene was  
10 integrated into the *Lemnaceae* chromosome adjacent to endogenous *Lemnaceae* regulatory sequences. As a result of random integration of the *uidA* gene, variability in the level of GUS expression would be expected among different transformation events. The results of the experiment show 3 different intensities of GUS blue stain, the most intense of which matches  
15 the typical strong intensity found in transformed plants under the 35S-CaMV promoter.

Table 13 summarizes the results for over 12,000 plants in 4 different experiments (each with 3 different strains of *A. tumefaciens*) which were scored for GUS staining 10 days after co-cultivation. Four thousand  
20 plants were co-cultivated with *A. tumefaciens* GV3103 lacking a binary vector (Control). None stained blue, indicating no endogenous GUS activity. Four thousand plants were co-cultivated with *A. tumefaciens* GV3103 harboring a promoterless construct (pVCGUS). Up to 1.4% of the plants stained blue, indicating a highly significant level (versus Control) of  
25 integration of the promoterless *uidA* gene into the *Lemnaceae* chromosome adjacent to endogenous *Lemnaceae* regulatory sequences. Four thousand plants were co-cultivated with *A. tumefaciens* GV3103 (pME504),  
harboring the *uidA* gene under the control of the 35S-CaMV promoter. Twenty eight percent of plants stained blue, indicating a relatively high level  
30 of expression using this heterologus promoter.

The method involved co-cultivation of approximately 3000 *Spirodela punctata* plants (6 gr fresh weight) in each transformation experiment (1000 plants for each of 3 constructs). Plants were scored for

- 37 -

GUS expression 10 days after co-cultivation. The results were categorized according to the intensity of blue color: light (+), medium (++) and dark (+++).

The data in Table 13 show that the variability in intensity of GUS staining among plants co-cultivated with *A. tumefaciens* GV3103 (pVCGUS) was considerably higher than with those co-cultivated with *A. tumefaciens* GV3103 (pME504). The relatively low percentage and variability (versus Control) in intensity of GUS staining plants co-cultivated with the promoterless GUS construct are explained by random integration of this gene into the *S. punctata* chromosome and expression by various endogenous *Spirodela* promoters.

Table 13

GUS expression (No. of fronds)												
	pVC-GUS				pME504				No. Ti plasmid			
	+	++	+++	total	+	++	+++	total	+	++	+++	to- tal
I	7	3	2	12	0	14	231	245	0	0	0	0
II	1	1	3	5	0	10	271	281	0	0	0	0
III	9	5	0	14	0	6	201	206	0	0	0	0
IV	3	1	2	6	0	21	209	230	0	0	0	0
Sum				37				962				0
% (ave.)	0.9				24				0			

**Example 19: Development of an *Agrobacterium*-mediated, *in-planta*, non-chimeric transformation of *Lemnaceae* without *de novo* regeneration**

It was demonstrated that it is possible to develop a novel transformation system in *Lemnaceae* which does not require tissue culture or *in vitro* regeneration procedures. The procedures enable *in-planta*, direct meristem targeting of the transforming vehicle. In this novel system, a meristem is transformed and has the capability to continue growing and thus

form the next generation. Moreover, no selection pressure is needed to avoid the growth of non-meristematic tissue within the same *Lemnaceae* plant. Examples of the validity of this approach can be seen in the results of Examples 1, 4, 6 and 10 through 18.

5

**Example 20    Method for long-term maintenance of morphogenetic *Spirodela* calli**

Transient callus formation and short-term calus maintence was  
10 previously reported in 2 species of *Lemna* (Chang and Chiu, 1978a, 1978b). Using one of these authors' callusing media (Murashige and Skoog, 1962), supplemented with 3% sucrose, 1 mg/l 2IP and 10 mg/l 2,4-D), calli were obtained from *Spirodela punctata* but callus development was arrested within  
14 to 21 days. During this period, dramatic accumulation of starch  
15 (measured as an increase in iodine staining material) within the calli were observed. These calli bleached and eventually died, apparently due to starch poisoning. Long term maintenance is a prerequisite to transformation at the callus level since screening and/or selection steps are needed.

Conditions for long term maintenance of *Spirodela* calli were  
20 sought. Several different media (MS medium (Table 1), B-5 (Table 5), and concentrations of sucrose (3.0, 1.0, 0.5%) were studied in combination with a number of phytohormones (2IP; 2,4-D; Dicamba; BA, Zeatin). B-5 medium supplemented with a low concentration (1.0%) of sucrose was found to best promote long term maintenance of *Spirodela* calli under  
25 several hormonal combinations. The data in Tables 14, 15 and 16 summarize percentage callus formation and long term maintenance under several hormonal combinations. The ultimate method for callus formation and long term maintenance of morphogenetic *Spirodela* calli is given in Procedure VII. It uniquely demands a low concentration of sucrose  
30 combined with particular hormonal combinations. Using this procedure, green, growing calli were maintained for more than three months.

The method involved culturing separated *Spirodela punctata* var. Helgm fronds on B-5 medium supplemented with 1% sucrose and various

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concentrations of 2IP and 2,4-D as indicated. Callus formation was monitored after 8 weeks using a dissecting microscope. Calli were subcultured to fresh medium every 4 weeks. The results are shown in Table 14:

Table 14

2 IP (mg/l)	2,4-D (mg/l)	Callus formation <sup>a</sup> No. (%)	Long term maintenance <sup>b</sup> No. (%)
2	2	5 (10)	0 (0)
2	10	134 (90)	48 (34)
2	50	3 (15)	0 (0)
10	2	2 (6)	0 (0)
10	10	4 (10)	0 (0)
10	50	0 (0)	0 (0)
<sup>a</sup> Data scored after 8 weeks of culture			
<sup>b</sup> Data scored after 12 weeks of culture			

In another experiment separated *Spirodela punctata* fronds were cultured on B-5 medium supplemented with 1% sucrose and various concentrations of BA and Dicamba as indicated. Callus formation was monitored after 3 weeks using a dissecting microscope. Calli were subcultured to fresh medium every 3 weeks. The results are shown in Table 15:

Table 15

BA (mg/l)	Dicamba (mg/l)	Callus formation <sup>a</sup> No. (%)	Long term maintenance <sup>b</sup> No. (%)
2	2	0 (0)	0 (0)
2	10	13 (23)	0 (0)
2	50	175 (100)	151 (86)
10	2	0 (0)	0 (0)
10	10	0 (0)	0 (0)
10	50	0 (0)	0 (0)
<sup>a</sup> Data scored after 3 weeks of culture			
<sup>b</sup> Data scored after 6 weeks of culture			

- 40 -

In another experiment separated *Spirodela punctata* fronds were cultured on B-5 medium supplemented with 1% sucrose and various concentrations of Zeatin and Dicamba as indicated. Callus formation was monitored after 3 weeks using a dissecting microscope. Calli were subcultured to fresh medium every 3 weeks. The results are shown in Table 16:

Table 16

Zeatin (mg/l)	Dicamba (mg/l)	Callus formation <sup>a</sup> No. (%)	Long term maintenance <sup>b</sup> No. (%)
2	2	0 (0)	0 (0)
2	10	0 (0)	0 (0)
2	50	10 (80)	7 (70)
10	2	0 (0)	0 (0)
10	10	0 (0)	0 (0)
10	50	13 (76)	6 (46)
<sup>a</sup> Data scored after 3 weeks of culture			
<sup>b</sup> Data scored after 6 weeks of culture			

**Example 21: Method for producing highly-regenerative *Spirodela* calli**

The unique method for production of *Spirodela* calli is described in Procedure VII. By using this procedure in conjunction with Procedure VIII, regenerated *S. punctata* plants were efficiently obtained (Table 17 below). The combination of the two procedures represents the method for producing highly-regenerative *Spirodela* calli.

Calli of *Spirodela punctata* var. Helgm, maintained for 7 to 16 weeks on B-5 medium supplemented with 1% sucrose, 2 mg/l 2IP and 10 mg/l 2,4-D, were transferred to B-5 medium supplemented with 1% sucrose and different concentrations of 2IP. Regenerated plants were visually scored after 2 weeks.

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Table 17

2 IP	Calli	Regenerating calli	
(mg/l)	No.	No.	(%)
0	30	26	87
2	30	22	73
10	30	0	0

**Example 22: Method for rapid and highly-efficient regeneration of *Lemnaceae* plants from calli**

Regeneration of frond-like structures from calli of *Lemna*  
 5 *perpusilla* has been reported; however, the authors state that they did not  
 observe further development of these frond-like structures even after 2  
 months (Chang and Hsing, 1978). In a further study, regeneration of plants  
 from calli of *Lemna gibba* (Chang and Chiu, 1978) was reported. However,  
 the procedure required 2 months to obtain an asexual propagating plant  
 10 Chang and Chiu, 1978). Regeneration of *Spirodela* plants from calli has  
 never been reported. Using the novel methodology of the invention for  
 plant regeneration, intact, regenerated *S. punctata* plants were efficiently  
 obtained within 1-2 weeks. The uniquely rapid method for *Lemnaceae*  
 plant regeneration is given in Procedure VIII.

15

**Example 23: Method for rapid and highly-efficient regeneration of true-to-type *Spirodela* plants from calli**

Using the methodology for plant regeneration (Procedure VIII),  
 20 > 90% of the regenerated *S. punctata* plants visually appeared true-to-type  
 after 3 weeks of growth in SP medium. *S. punctata* plants, viewed after 3  
 months of growth under standard conditions, continued to appear true-to-  
 type. When compared with their parental progenitor with respect to growth  
 rate, size and frond morphology no significant differences were found.

25

**Example 24: Method for increasing genetic diversity through calli in regenerating *Spirodela* plants**

5           The ability to increase the genetic diversity in *Spirodela* is important since in several species, propagation is strictly vegetative (Landolt and Kandeler, 1987). Using the methodology for plant regeneration (Procedure VIII), several regenerated plants visually appeared aberrant and after 3 weeks of growth under standard conditions, continued to appear  
10 aberrant. When compared with their parental progenitor, significant differences were found in size (smaller), growth rate (slower) and morphology (frond shape).

**Example 25: Transformation of *Lemnaceae* calli by *Agrobacterium***

15           *Spirodela* calli were maintained on B-5 medium (Table 6) supplemented with 1.0% sucrose, 2 mg/l 2IP and 10 mg/l 2,4-D. Five hundred calli were co-cultivated with *Agrobacterium* harboring the pME504 plasmid. Following 2 days of co-cultivation, the calli were transferred to fresh medium supplemented with 30 mg/l kanamycin and 300 mg/ml  
20 carbenicillin. After 15 days, 488 calli were fully bleached. The remaining 12 green calli were transferred to fresh medium supplemented with 30 mg/l kanamycin. Three calli remained green following two additional subcultures (2 months) on fresh media containing kanamycin as above. Green calli which were maintained for more than two months on B-5 medium  
25 supplemented with 1.0% sucrose, 2 mg/l 2IP, 10 mg/l 2,4-D and 30 mg/l kanamycin were monitored. The results indicate that the persistent, green calli were resistant to kanamycin as a result of the expression of the introduced genes.

30           **Example 26: Production of transgenic calli from *Agrobacterium*-infected intact *Lemnaceae* plants**

*Spirodela punctata* plants were maintained in SP medium under standard growth conditions (Table 2). Using the standard transformation



procedure (Procedure III), plants were co-cultivated with *A. tumefaciens* strain EHA105 harboring Ti plasmid pME504. Two days following the transformation the intact plants were cultured on B-5 medium supplemented with 10 mg/l Dicamba, 2 mg/l BA and 30 mg/l kanamycin (Procedure VII).

- 5 Two green, compact calli resistant to kanamycin developed from meristematic regions after 25 days. The green growing calli were dissected from the original tissue and further subcultured on fresh medium containing 30 mg/l kanamycin for an additional 20 days. Following this transfer as well, the 2 calli remained green. The results indicate that the persistent  
10 green calli were resistant to kanamycin as a result of the introduced genes.

**Example 27: Regeneration of transgenic *Lemnaceae* plants from *Agrobacterium*-transformed calli**

- 15 *Spirodela punctata* 8717 plants were maintained in SP medium under standard growth conditions (Table 2). Using the standard transformation procedure (Procedure III), plants were co-cultivated with *A. tumefaciens* strain EHA105 harboring Ti plasmid pME504. Two days following the transformation, the intact plants were cultured on B-5 medium  
20 supplemented with 10 mg/l Dicamba, 2 mg/l BA and 30 mg/l kanamycin (Procedure VII). Ten calli, resistant to kanamycin, developed from meristematic regions after 25 days. The green calli were transferred to B-5 medium supplemented with 1% sucrose, 2 mg/l 2IP and 30 mg/l kanamycin. A green regenerant plant was scored after 2 weeks. This plant was  
25 transferred to the same media approximately every 2 weeks for a period of 8 months, giving rise to numerous kanamycin-resistant vegetative offsprings, hereafter designated as clone ME11. Clone ME11 has been propagated as green and kanamycin resistant in the above media for more than 65  
generations.

- 30 The ability to obtain kanamycin resistant calli has been demonstrated either by production of transgenic calli from *Agrobacterium*-infected *Lemnaceae* plants (Example 25), or by production of transgenic calli from *Agrobacterium*-infected *Lemnaceae* calli (Example 26). Since kanamycin

resistant calli can be produced and true-to-type *Lemnaceae* plants can be efficiently and readily regenerated from calli (Procedure VIII), the technology for producing transformed *Lemnaceae* plants originating from antibiotic resistant calli has been demonstrated.

5

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## CLAIMS:

1. A genetically stable, transformed *Lemnaceae* plant and progeny thereof.
- 5 2. A transformed *Lemnaceae* plant according to Claim 1, of the genera *Spirodela*, *Lemna* and *Wolffia*.
3. A transformed *Lemnaceae* plant according to Claim 2, being *Spirodela punctata* of strain 8717.
4. An antibiotic resistant transformed *Lemnaceae* plant according to  
10 Claims 1 to 3.
5. A transformed *Lemnaceae* plant according to Claim 4, being resistant to kanamycin.
6. A herbicide resistant transformed *Lemnaceae* plant according to Claims 1 to 3.
- 15 7. A transformed *Lemnaceae* plant according to Claim 4, being resistant to BASTA 20.
8. A transformed *Lemnaceae* plant according to Claims 1 to 7, capable of expressing two or more foreign genes.
9. Use of the plant according to Claim 1, for the production of  
20 chemical or biological products.
10. Use according to Claim 9, for the production of polypeptides, proteins, carbohydrates, lipids, alkaloids, pigments or vitamins.
11. A chemical or biological product obtained by the use according to Claim 9 or 10.
- 25 12. A method for the stable genetic transformation of *Lemnaceae* plants which comprises: incubating *Lemnaceae* plants and/or tissue with *Agrobacterium* cells containing a transforming DNA molecule, whereby cells in said plant tissue become stably transformed with said DNA.
13. A method according to Claim 12, wherein the *Agrobacterium*  
30 cells are capable of specifically targeting the plant's meristematic tissue.

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14. A method according to Claim 13, wherein the *Agrobacterium* cells are *A. tumefaciens* strains EHA105, EHA101 and GVE3103.
15. A method according to Claim 12, wherein the *Agrobacterium* cells are capable of targeting wounded regions in the plant.
- 5 16. A method according to Claim 15, wherein the *Agrobacterium* is *A. tumefaciens* strains LBA4404 and C58.
17. A method according to Claims 12 to 16, wherein during the incubation of the *Lemnaceae* plant tissue with the *Agrobacterium* cells vacuum infiltration is applied.
- 10 18. A method according to Claim 12, wherein prior to incubation of the *Lemnaceae* plant tissue with the *Agrobacterium* cells the plant's meristematic zone is exposed by removal of the daughter fronds.
19. A method for the genetic transformation of a plant comprising: cutting the plant into particles of a size such that they still contain undamaged meristematic tissue capable of developing into full plants; incubating  
15 said particles with *Agrobacterium* cells containing transforming DNA molecules, whereby said transforming DNA is introduced into meristematic cells in said particles; and producing transformed plants from the transformed meristematic tissue.
- 20 20. A method according to Claim 19, wherein the plant is a *Lemnaceae* plant.
21. A method according to Claim 19 or 20, wherein the particles have an average diameter above about 150  $\mu\text{m}$ .
22. A method according to Claim 21, wherein the particles have an  
25 average diameter of about 150  $\mu\text{m}$  - 750  $\mu\text{m}$ .
- ~~23. A method for the stable genetic transformation of a *Lemnaceae* plant comprising microinjecting *Agrobacterium* cells containing a transforming DNA into the meristematic zone of the plant, whereby the meristemic tissue becomes stably transformed with said DNA.~~
- 30 24. A method according to Claim 23, carried out *in planta*.

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25. A method for the *in planta* transformation of *Lemnaceae* plants comprising:

- i. exposing the plant's meristematic zone by removal of the daughter fronds;
- 5 ii. incubating the plant with *Agrobacterium* cells capable of targeting to the meristemic tissue.

26. A method according to Claim 25, wherein the *Agrobacterium* cells are *A. tumefaciens* strains EHA105, EHA101 and GVE3103.

27. A method according to any one of Claims 12 to 26, wherein the  
10 *Agrobacterium* cells are brought into contact, prior or during the transformation method, with a booster medium capable of enhancing the *Agrobacterium* cell's virulence.

28. A method according to any one of Claims 12 to 26 wherein the transformation process takes place in a media having a pH below about 5.2.

15 29. A method according to Claim 28, wherein the booster medium further comprises a fresh cell suspension obtained from a dicotyledonous plant.

30. A method according to Claims 28 or 29, wherein the fresh cell suspension is at a concentration of 1-10% (w/v).

20 31. A method according to Claims 28 to 30, further comprising caffeine at a concentration of 100-500 mg per liter of medium.

32. A method according to any one of Claims 28 to 31, wherein the fresh cell suspension of a dicotyledonous plant is obtained from the family of *Solanaceae*.

~~25 33. A method according to any one of Claims 26 to 32, wherein the medium is a plant culture medium having a pH of about 3.5 to 4.2, and comprising 1-10% (w/v) of fresh cell suspension of *Nicotiana tabacum* and 100-500 mg per liter of medium caffeine.~~

30 34. A method according to Claim 27, wherein the booster medium comprises a *Lemnaceae* plant extract.

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35. A method according to Claim 34, wherein the *Lemnaceae* plant extracts are *Spirodela punctata* extracts.
36. A transformed *Lemnaceae* plant obtained by the method of any one of Claims 12 to 35.
- 5 37. A booster medium for enhancing *Agrobacterium* cell's virulence comprising plant tissue culture at a pH below about 5.2.
38. A booster medium according to Claim 37, further comprising a fresh cell suspension of a dicotyledonous plant.
39. A booster medium according to Claim 38, wherein the fresh cell  
10 suspension is at a concentration of 1-10% (w/v).
40. A booster medium according to any of Claims 37 to 39, further comprising caffeine at a concentration of 100-500 mg per liter of medium.
41. A booster medium according to any of Claims 37 to 40, wherein the fresh cell suspension is of plants from the family of *Solanaceae*.
- 15 42. A booster medium according to any of Claim 37 to 41, comprising plant growth medium at a pH of above 3.5 to 4.2, 1-10% (w/v) of fresh cell suspension of *Nicotiana tabacum*, and 100-500 mg per liter of medium caffeine.
43. A booster medium for enhancing *Agrobacterium* cell's virulence  
20 comprising an extract from *Lemnaceae* plants.
44. A booster medium according to Claim 41, comprising extracts of *Spirodela punctata* plants.
45. A method for maintaining morphogenetic *Lemnaceae* calli for long-periods of time comprising culturing the calli in a medium having a  
25 low level of sucrose.
- ~~46. A method according to Claim 45, wherein the sucrose level is less than 1.5%.~~
47. A method for the regeneration of plants from calli wherein the plant's growth medium has sucrose levels below 1.5% and comprises: B5,  
30 minerals and organic compounds.



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48. A method for the production of highly regenerative calli, wherein the calli's growth medium has sucrose levels below 1.5% and comprises B5, minerals and organic compounds.

5 49. A method according to Claim 47 or 48, wherein the growth media further comprises phytohormones.

50. A method for the production of highly regenerative calli, wherein the calli's growth medium has sucrose levels below 1.5% and comprises B5, minerals, organic compounds and selection agents.

10 51. A method according to Claim 50, wherein the selection agents are selected from the group consisting of: antibiotics, herbicides, or metabolic inhibitors.

52. A method for the production of stable transformed plants, wherein the growth media has sucrose levels below 1.5% and comprises B5, minerals and organic compounds.

15 53. A method according to Claim 52, wherein the growth media further comprises phytohormones.

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## A B S T R A C T

The present invention concerns genetically stable transformed *Lemnaceae* plants and methods for their transformation by *Agrobacterium* cells. The present invention further concerns a method for regeneration of plants from *calli*, utilizing low sucrose media. The present invention further  
5 concerns booster media for use in the above methods.

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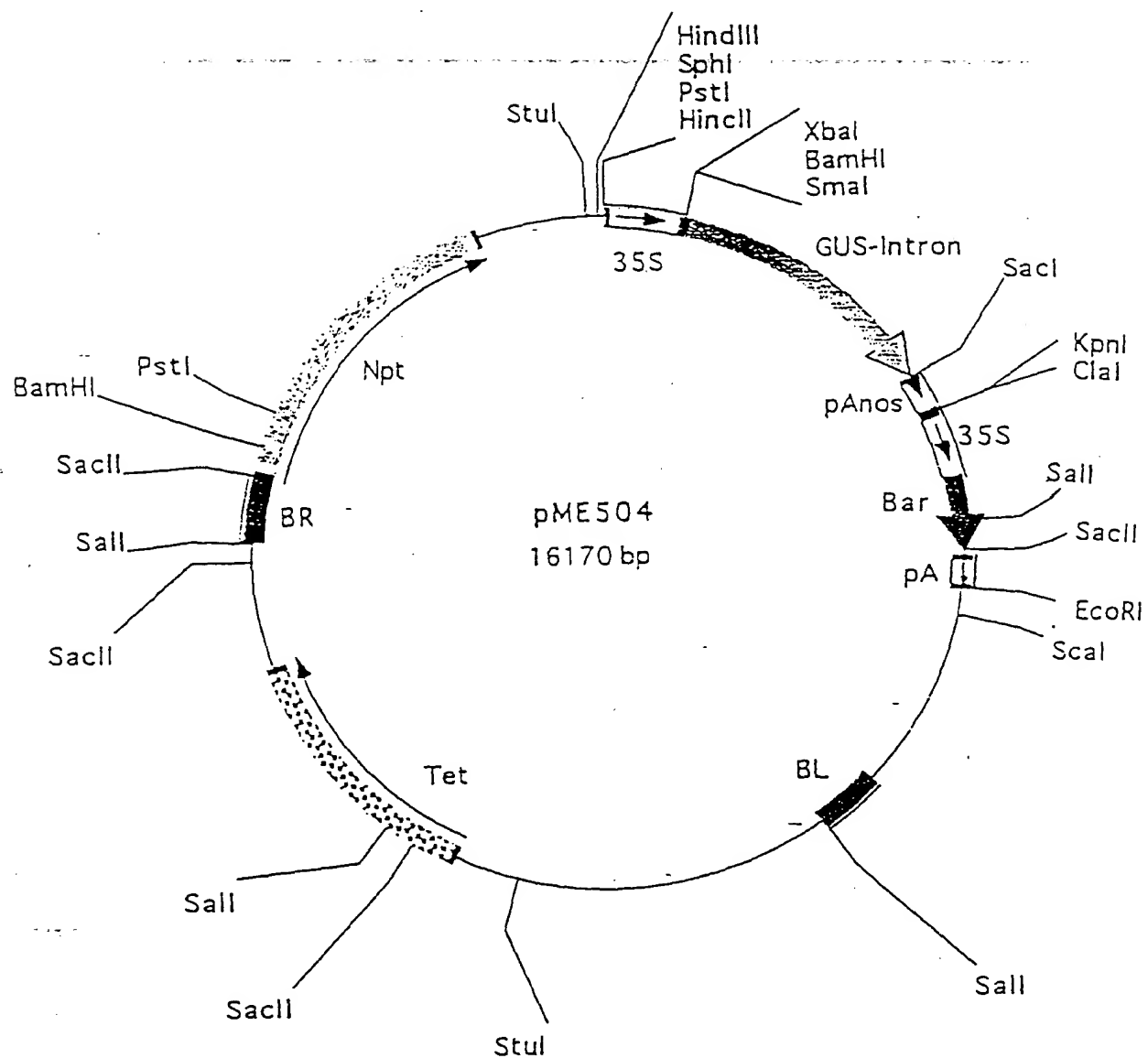


Figure 1

